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1 **Genomic identification of cryptic susceptibility to penicillins and β -lactamase**
2 **inhibitors in methicillin-resistant *Staphylococcus aureus***

3

4 Ewan M. Harrison^{1,2,3¶*}, Xiaoliang Ba^{4¶}, Francesc Coll⁵, Beth Blane², Olivier Restif⁴,
5 Henry Carvell⁶, Claudio U. Köser⁷, Dorota Jamroz¹, Sandra Reuter⁸ Andrew
6 Lovering⁹, Nicholas Gleadhall², Katherine L. Bellis², Anne-Catrin Uhlemann
7 ¹⁰, Franklin D. Lowy¹⁰, Ruth C. Massey⁶, Inês R. Grilo¹¹, Rita Sobral¹¹, Jesper
8 Larsen¹², Anders Rhod Larsen¹², Carina Vingsbo Lundberg¹² Julian Parkhill¹, Gavin
9 K. Paterson¹³, Matthew T.G. Holden¹⁴, Sharon J. Peacock^{1,2,5§}, Mark A. Holmes^{4§}

10 Wellcome Sanger Institute, Hinxton, United Kingdom¹

11 University of Cambridge, Department of Medicine, Cambridge, United Kingdom²

12 University of Cambridge, Department of Public Health and Primary Care, Cambridge,
13 United Kingdom³

14 University of Cambridge, Department of Veterinary Medicine, Cambridge, United
15 Kingdom⁴

16 London School of Hygiene & Tropical Medicine, London, United Kingdom⁵

17 University of Bristol, School of Cellular and Molecular Medicine, Bristol, United
18 Kingdom⁶

19 University of Cambridge, Department of Genetics, Cambridge, United Kingdom⁷

20 Medical Center – University of Freiburg, Institute for Infection Prevention and
21 Hospital Epidemiology, Freiburg, Germany⁸

22 University of Birmingham, Institute of Microbiology and Infection and School of
23 Biosciences, Birmingham, United Kingdom⁹

24 Columbia University, Division of Infectious Diseases, Department of Medicine, New
25 York, New York, USA.¹⁰

26 UCIBIO@REQUIMTE, Departamento de Ciências da Vida, Faculdade de Ciências e
27 Tecnologia, Universidade Nova de Lisboa, Lisboa, Portugal¹¹

28 Statens Serum Institut, Department of Bacteria, Parasites & Fungi, Copenhagen,
29 Denmark¹²

30 Royal (Dick) School of Veterinary Studies, University of Edinburgh and The Roslin
31 Institute, United Kingdom¹³

32 School of Medicine, University of St Andrews, St Andrews, United Kingdom¹⁴

33 *Corresponding author - Ewan M. Harrison (eh6[at]cam.ac.uk)

[¶]Contributed equally [§]Co-senior author

Abstract

Antibiotic resistance in bacterial pathogens threatens the future of modern medicine. One such resistant pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to nearly all β -lactam antibiotics, limiting treatment options. Here, we show that a significant proportion of MRSA isolates from different lineages, including the epidemic USA300 lineage, are susceptible to penicillins when used in combination with β -lactamase inhibitors such as clavulanic acid. Susceptibility is mediated by a combination of two different mutations in the *mecA* promoter region that lowers *mecA*-encoded penicillin binding protein 2a (PBP2a) expression, and in the majority of isolates by either one of two substitutions in PBP2a (E246G or M122I) that increase the affinity of PBP2a for penicillin in the presence of clavulanic acid. Treatment of *S. aureus* infections in wax moth and mouse models demonstrate that penicillin/ β -lactamase inhibitor susceptibility can be exploited as an effective therapeutic choice for 'susceptible' MRSA infection. Finally, we show that isolates with the PBP2a E246G substitution have a growth advantage in the presence of penicillin, but the absence of clavulanic acid, which suggests that penicillin/ β -lactamase susceptibility is an example of collateral sensitivity (resistance to one antibiotic increases sensitivity to another). Our findings suggest that widely available and currently disregarded antibiotics could be effective in a significant proportion of MRSA infections.

61 **Introduction**

62 The β -lactam family of antibiotics, is one of the most widely used and clinically
63 important groups of antibiotics ¹. Resistance to β -lactam antibiotics in
64 *Staphylococcus aureus* is either mediated by the acquisition of the *blaZ* gene
65 encoding a β -lactamase, or in methicillin-resistant *S. aureus* (MRSA) from the
66 acquisition of an alternative penicillin binding protein 2a (PBP2a) with a low affinity
67 for β -lactam antibiotics, resulting in resistance to most β -lactams ². PBP2a is
68 encoded by *mecA* or *mecC* (PBP2a'/c) carried on a mobile genetic element known as
69 a staphylococcal cassette chromosome *mec* (SCC*mec*) ^{3,4}, and is regulated by two
70 independent regulatory systems (*mecI-mecR-mecR2* and *blaI-blaR*) and multiple
71 chromosomal genes ⁵.

72
73 Countering resistance to β -lactams was first achieved by the development of β -
74 lactamase resistant β -lactams such as methicillin ⁶, and subsequently by the
75 development of β -lactamase inhibitors ⁷. Unfortunately, MRSA is resistant to both
76 methicillin antibiotics and insensitive to β -lactamase inhibitors. However, in the early
77 1990s, β -lactams and β -lactamase inhibitors were tested against MRSA ⁸⁻¹¹, with
78 success both *in vivo* and clinically ¹².

79
80 Recently, different approaches to restore susceptibility to β -lactams have been
81 investigated, including using multiple antibiotics to exploit collateral sensitivity
82 (resistance to one antibiotic increases sensitivity to another) ¹³⁻¹⁵. We recently
83 demonstrated that the small subset of MRSA isolates with the *mecC* gene are
84 susceptible to penicillins and β -lactamase inhibitors, as the *mecC* encoded PBP2c
85 does not mediate resistance to penicillins ¹⁶. Here, we demonstrate that
86 unexpectedly, a significant proportion of all clinical *mecA*-positive MRSA isolates
87 from diverse lineages are susceptible to penicillins and β -lactamase inhibitors. These

88 findings could provide the basis of new treatment options for susceptible MRSA
89 infections using already licenced antibiotics.

90

91 **Results**

92 **Penicillin/ β -lactamase inhibitor-susceptible MRSA**

93 As part of an earlier study ¹⁶, we identified an *mecA*-positive MRSA isolate that
94 exhibited increased susceptibility to penicillin in the presence of clavulanic acid (a β -
95 lactamase inhibitor) (Fig 1a). We screened a selection of whole genome sequenced
96 (WGS) MRSA isolates (n = 110) from different clinically relevant clonal lineages for
97 the same increase in susceptibility (≥ 10 mm increase in the zone of inhibition in the
98 presence of 15 $\mu\text{g/ml}$ clavulanic acid, compared to penicillin alone). Fifty-five (50.0%)
99 isolates from different lineages were susceptible to penicillin-clavulanic acid
100 (Supplementary Table 1). We determined the minimum inhibitory concentration (MIC)
101 for penicillin for a subset of isolates, which showed that the MIC was reduced below
102 the European Committee on Antimicrobial Susceptibility Testing (EUCAST) / Clinical
103 and Laboratory Standards Institute (CLSI) breakpoint (≤ 0.125 $\mu\text{g/ml}$) ^{17,18} in ten of
104 the fourteen susceptible isolates, and none of the ten resistant isolates (Fig. 1b). Two
105 other β -lactam inhibitors, sulbactam and tazobactam also increased susceptibility to
106 penicillin (Supplementary Fig. 1), suggesting that the effect of clavulanic acid was
107 due to inhibition of the staphylococcal β -lactamase. Next, we evaluated if the
108 increase in susceptibility was penicillin-specific by screening isolates against different
109 β -lactam antibiotics in the presence of clavulanic acid (Fig. 1c and d). This revealed
110 that the penicillin-clavulanic acid susceptible isolates showed the greatest increase in
111 susceptibility to penicillins (benzyl- and aminopenicillins), (Fig. 1d) and were broadly
112 more susceptible to cephalosporins (except ceftaroline) and carbapenems than the
113 resistant isolates (Fig 1c). In contrast, the presence of clavulanic acid produced only
114 minor increases in susceptibility in the resistant isolates (Fig 1c).

115 Substitutions in PBP2a mediate increased penicillin susceptibility

116 Benzyl- and aminopenicillins bind reasonably well to PBP2a in comparison to the
117 isoxazolyl penicillins (oxacillin) and cephalosporins¹⁹. As amino acid substitutions in
118 PBP2a mediate resistance to fifth-generation cephalosporins²⁰, in the absence β -
119 lactamase activity, we hypothesised that inversely, increased susceptibility to
120 penicillins might be mediated by PBP2a substitutions. We examined the PBP2a
121 amino acid sequences of the 110 isolates (Supplementary Table 1). This revealed
122 that 80.0% (44/55) of the penicillin-clavulanic acid susceptible isolates had either a
123 E246G (PBP2a^{246G}) (n=37) or a M122I (PBP2a^{122I}) (n=7) substitution in the allosteric
124 domain of PBP2a²¹. By contrast, only 29.1% (16/55) of the resistant isolates had the
125 PBP2a^{246G} substitution. Phylogenetic analysis of PBP2a indicated that 246E is
126 present in the *S. aureus* COL genome (M122I was absent), an early MRSA strain
127 isolated in the 1960s, with origins in the 1940s^{22,23}, suggesting this might be the
128 ancestral or 'wildtype' form (Supplementary Fig. 2).

129

130 Next, we tested the effect of the PBP2a substitutions experimentally. We deleted
131 *mecA* (PBP2a) in isolates from two *S. aureus* lineages: ST398 (EC139²⁴) and
132 USA300/ST8 (BCVA289²⁵) (both PBP2a^{246G} and *blaZ* positive) and introduced
133 plasmid-borne copies of three *mecA* alleles: the putatively resistant 'wildtype'
134 *mecA*_{246E} and the two alleles associated with susceptibility: *mecA*_{122I} and *mecA*_{246G}.
135 All the complemented strains were resistant to penicillin alone (MICs >20 μ g/ml -
136 breakpoint >0.125 μ g/ml) (Fig. 2a). For penicillin-clavulanic acid, the *mecA*_{246E} strains
137 were resistant with MICs of 1 μ g/ml (Fig. 2a). While the strains with *mecA*_{246G} were
138 susceptible, with MICs of 0.125 μ g/ml. Similarly strains with *mecA*_{122I} had MICs of
139 0.125 and 0.25 μ g/ml for USA300 and ST398 backgrounds, respectively. No
140 difference in susceptibility was seen for cefoxitin (a cephalosporin), confirming that

the effect of the substitutions was limited to penicillin susceptibility (Supplementary Table 2).

A combination of *mecA* promoter mutations and PBP2a substitutions mediate susceptibility

We reasoned that the PBP2a substitutions might cause an increase in the affinity of PBP2a for penicillin. We tested PBP2a^{246E}, PBP2a^{246G} and PBP2a^{122I} in a bocillin competition assay to determine their relative binding affinities for penicillin (Fig 2b). This identified that all three variants had a similar affinity for penicillin, with 50% inhibitory concentrations (IC₅₀) of 10.63 µg/ml (standard error of the mean ± 0.53) for PBP2a^{246G} and 12.11(± 1.07) for PBP2a^{122I} and 9.06 (± 2.70) for PBP2a^{246E} (Fig 2b). As susceptibility testing had been carried out in the presence of clavulanic acid, we repeated the bocillin binding assays with 15 µg/ml clavulanic acid. In the presence of clavulanic acid, the wildtype PBP2a^{246E} had a two-fold higher IC₅₀ of 32.07 (± 8.38) compared to that PBP2a^{246G} 16.22 ± 3.19) and PBP2a^{122I} (11.73 ± 4.50), which were virtually unaffected (Fig 2c).

To confirm the consistency of association between the two PBP2a substitutions and phenotypic susceptibility, we determined the penicillin and penicillin-clavulanic acid MICs for 274 WGS isolates from a range of *S. aureus* lineages (Supplementary Table 3). We then combined the data with the original 24 isolates with MIC data (Fig. 1b) and plotted the frequency distributions of the penicillin and penicillin-clavulanic acid MICs (Fig. 2d and e). In the absence of a clinical breakpoint for penicillin-clavulanic acid we determined a tentative epidemiological (wildtype) cut-off (ECOFF) using a statistical method based on mixture models^{26,27}. This supported setting the ECOFF between 2.0 and 3.0 µg/ml (2.449, rounded down to 2.0 µg/ml) for penicillin in the presence of 15 µg/ml clavulanic acid (Fig 2d). The same method supported an ECOFF between 0.19 and 0.25 µg/ml (0.21, rounded down to 0.19 µg/ml) for

169 penicillin alone, which is within one doubling dilution of the EUCAST clinical
 170 breakpoint (Fig 2e)¹⁸. Using the ECOFF of ≤ 2.0 $\mu\text{g/ml}$ as the cut-off, 213 (71.5%)
 171 isolates were classified as susceptible to penicillin-clavulanic acid, of which only 176
 172 (82.6%) had either a 246G or 122I substitution. This indicated that 37 (17.4%)
 173 isolates were susceptible despite having no PBP2a substitution. Conversely, only 37
 174 (43.5%) of the resistant isolates (MIC > 2 $\mu\text{g/ml}$) had a 246G substitution.
 175
 176 Incomplete congruence between PBP2a substitutions and penicillin-clavulanic acid
 177 susceptibility led us to search for further mutations involved in susceptibility. We
 178 hypothesised that higher levels of PBP2a expression might overcome the effect of
 179 the PBP2a substitutions. We screened the same 298 isolates for mutations in the
 180 *mecA* promoter region, this identified two mutations that correlated with susceptibility.
 181 The first mutation was a G to T transversion in the *mecA* ribosomal binding site
 182 (RBS), seven nucleotides upstream of *mecA* start codon (Fig 3a). Isolates with the
 183 *mecA*[-7]:T allele had a median penicillin-clavulanic acid MIC of 0.125 $\mu\text{g/ml}$ (range
 184 $< 0.016 - 6$) compared to a median of 8 $\mu\text{g/ml}$ (range: 0.023 – 96) for isolates with the
 185 ‘wildtype’ *mecA*[-7]:G. Previous work has demonstrated that despite being in the
 186 RBS, the T allele results in lower *mecA* transcript and PBP2a expression levels²⁸.
 187 We compared relative levels of *mecA* expression by reverse transcription quantitative
 188 polymerase chain reaction (RT-qPCR) in isolates from phylogenetically separate
 189 lineages with the *mecA*[-7]:G (n=7) allele to isolates with the *mecA*[-7]:T allele (n=6)
 190 (Fig 3b). Isolates with the T allele had a statistically significantly lower relative
 191 expression (mean relative expression: 3.24) than isolates with the G allele (mean:
 192 7.00) (P=0.0048) (Fig 3b). The second mutation was a C to T transition in the *mecA* -
 193 10 box, 33 nucleotides upstream of the *mecA* start codon (*mecA*[-33]:T) (Fig 3a).
 194 Isolates with this mutation had a median penicillin-clavulanic acid MIC of 0.047 $\mu\text{g/ml}$
 195 (range: < 0.016 to 0.125). The C to T transition causes the generation of a perfect

196 palindrome within the MecI-BlaI binding site, which lowers *mecA* transcript and
 197 PBP2a expression levels ^{28,29}. Three isolates from distinct lineages with the *mecA*[-
 198 33]:T mutation had a mean relative expression rate of 0.27, suggesting the C to T
 199 transition results in very low levels of *mecA* expression in the tested isolates (Fig 3b).
 200
 201 When considering the two promoter mutations (*mecA*[-7]:G-T and *mecA*[-33]:C-T)
 202 together with the two PBP2a substitutions (PBP2a^{246G} and PBP2a^{122I}) (Fig 3a) we
 203 identified six genotypes. These were used to annotate the MIC distributions for
 204 penicillin and penicillin-clavulanic acid (Fig 3c and d). For penicillin-clavulanic acid,
 205 the genotypes split clearly into the bimodal distribution (Fig 3c). The majority of
 206 isolates with *mecA*[-7]:G (henceforth: Resistant 1) and *mecA*[-7]:G | E246G
 207 (Resistant 2) genotypes were found in the modal peak to the right with MICs above
 208 the ECOFF of ≥ 2 $\mu\text{g/ml}$ (Fig 3c). The majority of isolates with the other four
 209 genotypes: *mecA*[-7]:G-T (henceforth: Susceptible 1), *mecA*[-7]:G-T | E246G
 210 (Susceptible 2), *mecA*[-33]:C-T | *mecA*[-7]:G | E246G (Susceptible 3) and *mecA*[-
 211 7]:G | M122I (Susceptible 4) were located in the modal peak to the left with MICs
 212 below the ECOFF (≤ 2.0 $\mu\text{g.ml}$). Isolates with susceptible genotypes had lower
 213 penicillin MICs in the absence of clavulanic acid than those with resistant genotypes
 214 (Fig 3c and d). The use of the six genotypes to predict susceptibility using the
 215 ECOFF as breakpoint was accurate in 94.6% (282/298) of isolates, with a 0.34%
 216 (1/298) very major error (VME) rate (defined as isolates that were phenotypically
 217 resistant but genotypically predicted to be susceptible) and a 5.0% (15/298) major
 218 error (ME) rate (phenotypically susceptible, genotypically predicted resistant).
 219
 220 Finally, we investigated if the presence of the six different types of class A
 221 staphylococcal β -lactamases (types A-F), or *blaZ* expression levels might affect
 222 penicillin-clavulanic acid susceptibility ¹⁶. There was no association between the β -
 223 lactamase type and susceptibility that wasn't better explained by the six genotypes

224 (Supplementary Table 3 and Fig. 3a). Nor was there any significant association ($P=$
225 0.43) between *blaZ* expression and susceptibility in twenty isolates (11 susceptible, 9
226 resistant) as measured by RT-qPCR (Supplementary Fig. 3b).

227

228 **Clinical prevalence and epidemiology**

229 We sought to quantify the frequency of penicillin-clavulanic acid susceptibility in
230 clinical MRSA isolates. We tested a collection of 270 *S. aureus* isolates (MRSA, $n =$
231 218; MSSA isolates, $n = 52$) collected by the Danish Staphylococcus Reference
232 Laboratory as part of bacteraemia surveillance³⁰ (Supplementary Table 4). Isolates
233 were classified as resistant or susceptible based on the ECOFF of ≤ 2.0 $\mu\text{g/ml}$
234 penicillin in the presence of 15 $\mu\text{g/ml}$ of clavulanic acid. All MSSA isolates, and
235 84.9% (185/218) of the MRSA isolates had an MIC below the ECOFF. The MRSA
236 isolates were from a variety of lineages as inferred from *spa*-typing including clonal
237 complex (CC)1, CC5, CC8, CC30 and CC80. We then screened 2282 WGS MRSA
238 isolates from Cambridge, UK for the six genotypes (Fig. 4a)³¹. None of the isolates in
239 this collection had the Susceptible 4 genotype. Overall, 25.0% of isolates had one of
240 the three remaining putative susceptible genotypes (Fig 4a). The dominant UK
241 MRSA sequence type is ST22 (EMRSA-15), if the CC22 isolates (70.4% of isolates)
242 were excluded then 82.8% ($n=610$) had one of three susceptible genotypes (Fig. 4a).
243 Lineages with a high abundance of susceptible genotypes included CC1, CC5, CC8,
244 CC30 and CC59 (Fig. 4a). Importantly, 56 of the CC8 isolates (from 24 patients)
245 were USA300 which is the dominant clonal lineage in the United States (USA)³². All
246 56 USA300 isolates had the Susceptible 2 genotype and a penicillin-clavulanic acid
247 MIC below the ECOFF (Supplementary Table 3). We performed a phylogenetic
248 analysis of 580 CC8 isolates, including 485 USA300 isolates (457 MRSA, 28 MSSA)
249 from across the USA^{33,34}. All 457 of the USA300 MRSA isolates had the Susceptible
250 2 (*mecA*[-7]:G-T | E246G) genotype carried on a *SCCmec* type IVa ($n=455$) or IVb

251 element (n=1) (one isolate was non-SCCmec typeable), suggesting that the majority
252 of the USA300 population is susceptible to penicillin-clavulanic acid (Fig 4b). We then
253 screened 23 USA300 isolates, distributed across the phylogeny (isolated in New
254 York ³⁴) for penicillin-clavulanic acid susceptibility (Fig. 4b). All had an MIC below the
255 ECOFF (<2 µg/ml) (Supplementary Table 5).

256

257 **Heterogeneity of susceptible populations**

258 β-lactams resistance in most MRSA is heterogenous, meaning that while most cells
259 in a population have low MICs, a fraction (10^{-4} – 10^{-8}) can survive at much higher MICs
260 ³⁵. We tested representative isolates for all six penicillin-clavulanic acid susceptibility
261 genotypes in a population analysis for their susceptibility profile to penicillin-
262 clavulanic acid. Isolates representing the four susceptible genotypes all displayed
263 heterogenous resistance to penicillin-clavulanic acid (Fig 5a). CFUs were drastically
264 reduced at low concentrations of penicillin (0.12 to 1 µg/ml) and susceptible isolates
265 had a median population MIC of 12 µg/ml (range 0.48 to 32) (Fig 5a). Notably, for
266 four isolates from three different genotypes (Susceptible 1, 3, and 4) the entire
267 population was completely inhibited by 4 µg/ml. The remainder of susceptible
268 isolates had 'highly resistant cells' (cells capable of growing in concentrations greater
269 than the ECOFF of ≤2 µg/ml) present at a frequency of 10^{-5} – 10^{-8} . In contrast,
270 resistant isolates displayed homogenous resistance to penicillin-clavulanic acid (Fig
271 5a). CFUs of resistant isolates were unaffected at the lower penicillin concentration
272 range (0.12 to 1 µg/ml) and had a higher median population MIC of 64 µg/ml (range
273 64 to 128), greater than the susceptible isolates (Fig. 5a). Highly resistant cells (MIC
274 ≥4 µg/ml) were also present at higher frequencies (1 – 10^{-4}) than susceptible isolates.

275

276 **Penicillin-clavulanic acid is effective for MRSA treatment *in vivo***

277 We next sought to demonstrate efficacy in physiologically relevant infection models in
278 which highly resistant cells would be present. First, we used a wax moth larvae

279 model of infection, larvae were infected with four different MRSA isolates, two with a
280 resistant genotype (both ST22: Resistant 1) and two with a susceptible genotype
281 (ST398 and ST8 (USA300): Susceptible 2). Treatment at approximate human
282 dosages was with penicillin, penicillin-clavulanic acid, amoxicillin, amoxicillin-
283 clavulanic acid, vancomycin or PBS (vehicle). Only vancomycin offered any
284 improvement in survival for the two resistant isolates (Fig 5b and c). In contrast, for
285 the two susceptible isolates both penicillin-clavulanic acid and amoxicillin-clavulanic
286 acid increased survival in comparison to penicillin or amoxicillin alone, increasing the
287 median survival times by 40 hours (Fig 5d and e). Given that amoxicillin-clavulanic
288 acid is clinically available, we further tested its efficacy in a more physiologically
289 relevant, higher infective dose (1×10^7 CFU) murine thigh infection model, with
290 approximate human dosages using a USA300 strain (strain: MRSA 43484³⁶
291 (Susceptible 2, penicillin-clavulanic acid MIC: 0.19 µg/ml, population analysis:
292 Supplementary Fig. 4), USA300 phylogeny: Fig. 4b). Treatment with amoxicillin alone
293 in a dose range of 10 – 100 mg/kg did not reduce the bacterial loads compared to
294 vehicle treatment, whereas 100 mg/kg amoxicillin in combination with 20 mg/kg
295 clavulanic acid significantly reduced the bacterial loads to a similar level as 40 mg/kg
296 vancomycin (Dunnett's multiple comparisons test, $p < 0.0001$) (Fig. 5f). Demonstrating
297 the efficacy of amoxicillin-clavulanic acid as a treatment in a high dose infection
298 model.

299

300 **PBP2a substitutions provide a growth advantage in the presence of penicillin**

301 As the acquisition of *mecA* (PBP2a) in a *SCCmec* element can exhibit significant
302 fitness costs³⁷, and affect toxicity³⁸ and biofilm formation³⁹, we hypothesised that
303 the PBP2a substitutions might confer a fitness advantage. We found no significant
304 difference in biofilm formation or toxicity to human monocytic cells between any of
305 the three PBP2a variants in two strain backgrounds (Supplementary Fig. 6). We then
306 investigated the effect of the PBP2a substitutions on general fitness in three lineages

(ST22, USA300/ST8) and ST398) assayed by growth in a minimal medium, and in the presence of penicillin. In minimal medium, there were only minor differences (Fig 6a, c, e), with the USA300 strain (BCV289) complemented with *mecA*_{246G} growing marginally better in early exponential phase than *mecA*_{246E} or *mecA*_{122I} (Fig. 6a). In the ST22 (A75) background the *mecA*_{246E} strain grew slower during exponential growth than the other backgrounds (Fig 6e). In the presence of penicillin, in all three strain backgrounds the isolates complemented with *mecA*_{246G} grew better than the other two variants (Fig 6b, d and f). This was most pronounced in the ST398 background (EC139), in which *mecA*_{246G} strain grew considerably better in exponential phase, reached a higher optical density and grew with a reduced doubling time (dt) of 5.33 hours (95% confidence intervals (CI): 5.28 to 5.38) compared to either *mecA*_{246E} (dt: 6.65, 95% CI: 6.62 to 6.69) or *mecA*_{122I} (dt: 6.92, 95% CI: 6.89 to 6.96) (Fig 6d). This demonstrated that complementation with *mecA*_{246G} provided a growth advantage in the presence of penicillin, but the magnitude of this effect is influenced by strain background.

322

323 Discussion

324 We show that a significant proportion of clinical MRSA isolates are susceptible to a
325 combination of penicillins and a β -lactamase inhibitor. Susceptibility is due to one of
326 two different mutations in the *mecA* promoter region that both lower *mecA* (PBP2a)
327 expression, and in the majority of isolates, by an additional substitution in PBP2a
328 (E246G or M122I) that increases the affinity of PBP2a for penicillin in the presence of
329 clavulanic acid. It is not clear how clavulanic acid causes the increased binding
330 affinity of penicillin for PBP2a, as clavulanic acid binds to PBP2a poorly¹⁹. Modelling
331 of PBP2a shows that position 246 is located near the allosteric site but does not
332 indicate any clear mechanism for the increased affinity for penicillin (Supplementary
333 Fig.7 and Supplementary Discussion). In some isolates, a RBS mutation alone
334 appeared to be sufficient to mediate susceptibility, although given the complexity of

the regulation of β -lactam resistance in MRSA other genes might be involved ⁵. Crucially, the PBP2a 246G substitution provides a fitness benefit for growth in presence of penicillin, suggesting that susceptibility to penicillin and β -lactamase inhibitors is a likely a case of collateral sensitivity ⁴⁰, which evolved due to selective pressure for maintaining the balance between fitness and resistance.

In our susceptibility assays we used Iso-Sensitest media (ISA) rather than Müller-Hinton agar (MHA) as currently recommended by CLSI and EUCAST ^{18,19}, because ISA was the recommended media for penicillin by the British Society for Antimicrobial Chemotherapy (BSAC) at the beginning of the study ⁴¹. Comparison between susceptibility to penicillin-clavulanic acid on ISA and MHA revealed that a number of isolates that were susceptible on ISA remained resistant on MHA (Supplementary table 5), including isolates that responded to treatment *in vivo* (Fig 5b-e). This suggests that MHA is not the optimum media for the detection of susceptibility of penicillins and β -lactamase inhibitors, which is supported by a recent study which revealed that MHA failed to detect susceptibility to multiple antibiotic classes that were effective *in vivo* ⁴². Our data also highlights the risk of using a single antibiotic (e.g. cefoxitin for MRSA) to determine resistance to an entire antibiotic class, potentially missing unexpected susceptibilities.

Both *in vitro* and *in vivo*, penicillins and clavulanic acid were efficacious at physiologically achievable concentrations ⁴³. In the absence of a clinical breakpoint, pharmacokinetic-pharmacodynamic (PK-PD) breakpoints can be used to infer susceptibility ⁴⁴. The tentative ECOFF wildtype cut-off of ≤ 2.0 $\mu\text{g/ml}$ penicillin in the presence of 15 $\mu\text{g/ml}$ of clavulanic acid, lies in the intermediate susceptibility category (susceptible ≤ 0.25 $\mu\text{g/ml}$, resistant > 2 $\mu\text{g/ml}$) of the EUCAST PK-PD breakpoint ¹⁸. Large numbers of isolates had much lower MICs, and had amoxicillin-

362 clavulanic acid zone diameters greater than the breakpoint for other pathogenic
363 species (Supplementary Table 1, 3 and 4) ¹⁸. Previous studies have reported the
364 successful use of penicillins and β -lactamase inhibitors for the treatment of MRSA in
365 rabbits and rats, and for human infections ^{11,45-47}. This work, provides a mechanistic
366 explanation for efficacy in these studies, although there have been previous
367 conflicting reports ⁹. While it is unlikely that penicillins and β -lactamase inhibitor
368 combinations would be used as a monotherapy, they would be attractive additional
369 therapeutic option for hard-to-treat infections such as multidrug-resistant MRSA ⁴⁸,
370 particularly as β -lactams synergise with vancomycin and daptomycin ^{49,50}. PK/PD
371 modelling studies, including an assessment of highly resistant cells during treatment
372 is now needed to determine the optimum dosing strategy required for sustained
373 efficacy before appropriate clinical trial could be conducted.

374

375 Our findings demonstrate that cryptic susceptibilities to already licensed and
376 inexpensive antibiotics may emerge within constantly evolving bacterial populations,
377 which then can be exploited for the treatment of antibiotic resistant pathogens.

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389 **Material and methods**

390 **Media and culture conditions**

391 Bacterial strains and plasmids used in this study are described in Supplementary
392 Table 5 and 6. For routine culture, *Escherichia coli* (*E. coli*) was grown in Lysogeny
393 broth (LB) or on LB-agar (Oxoid, UK) at 37°C. *S. aureus* was grown on tryptone soy
394 agar (TSA), Columbia blood agar or in tryptone soy broth (TSB) (Oxoid, UK) at 28°C
395 or 37°C accordingly. *E. coli* and *S. aureus* media were supplemented with 10 µg/ml
396 chloramphenicol (Cm10) as appropriate. For growth curve studies, *S. aureus* strains
397 were grown in SSM9PR minimal medium (1 × M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂,
398 1% glucose, 1% casaminoacids, 1 mM Thiamine-HCl and 0.05 mM nicotinamide) at
399 37°C⁵¹.

400

401 **Isolate selection**

402 For the 110 sequenced isolates in Supplementary Table 1, isolates were selected
403 from sequenced isolates in our laboratory collections to provide a broad as possible
404 selection of isolates as possible from different clinically relevant lineages (Multilocus
405 sequence type (ST)1, 5, 8 22, 36, 45, 80, 88, 239, 398), with an obvious bias towards
406 lineages that dominate in the UK (e.g. 40 isolates from ST22, which is the dominant
407 lineage in the UK). For the additional 274 isolates that were combined with 24 of the
408 original isolates with MIC data (from the previous 110), we again tried to select
409 isolates from our laboratory collections that broadly covered a selection of clinically
410 relevant MRSA lineages including clonal complex (CC) 1, 5, 8 (including 56 UK
411 USA300 isolates), 22, 30, 45, 59, 72, 80, 97, 239, and 398 - amongst others). For
412 lineages with multiple isolates (CC1, n=25, CC5, n=29, CC22, n=91, CC59, n=18,
413 CC45, n=15, CC30, n=20) we used previously generated whole genome phylogenies
414³¹ to select isolates broadly across the phylogeny, as well as selecting isolates from
415 the same clades.

416

417 **Antimicrobial susceptibility testing**

418 Inocula were prepared by the growth method. At least four morphologically similar
419 colonies were touched with a sterile loop and transferred into Iso-Sensitest broth
420 (Oxoid, UK). Inoculated broth was incubated at 37°C with 200 rpm shaking until the
421 visible turbidity reached 0.5 McFarland standard. For disk diffusion and Etest method
422 testing, the 0.5 McFarland standard broth was diluted 1:10 in distilled water before
423 spreading onto agar plates. For Microbroth dilution for minimum inhibitory
424 concentrations (MIC), the broth was diluted 1:100 in Iso-Sensitest broth for
425 inoculation.

426

427 Disk diffusion susceptibility testing was carried out according to BSAC criteria (BSAC
428 Methods for Antimicrobial Susceptibility Testing, version 14, January 2015).

429 Temocillin disks were purchased from MAST group, UK. All other antibiotic disks
430 were purchased from Oxoid, UK. For the clavulanic acid assay, 15 µg/ml clavulanic
431 acid (Sigma-Aldrich, UK) was added to Iso-Sensitest agar (ISA) or Müller-Hinton agar
432 (MHA) (Oxoid, UK), as appropriate. After applying the antibiotic disks, all plates were
433 incubated at 35°C for 20 hours before inhibition zones were measured. Oxacillin disk
434 diffusion was also performed on MHA with 2% NaCl at 30°C for 24 hours. For disk
435 testing with clavulanic acid, susceptibility was defined as ≥10 mm increase of the
436 zone of inhibition in the presence of 15µg/ml clavulanic acid. For *mecA*
437 complemented strains, ISA was supplemented with 200 ng/ml anhydrotetracycline
438 (Atc) (Sigma-Aldrich, UK) to induce expression of *mecA* from pXB01, a modified
439 tetracycline-inducible expression vector pRMC2¹⁶. Microbroth dilution for minimum
440 inhibitory concentrations (MIC) was performed according to BSAC guidelines⁵². The
441 antibiotic ranges were prepared in Iso-Sensitest broth a step higher than the final
442 concentrations of 0.015-128 µg/ml for penicillin and 1-32 µg/ml for ceftiofur. A 96 well
443 cell culture plate (Greiner Bio-One, CELLSTAR®) was loaded with appropriate
444 antibiotic dilutions. For each test isolate, 75 µl of each antibiotic dilution were added

445 to a row of wells and 75 µl diluted test isolate culture was added into the wells. Each
446 isolate was tested in triplicate. The 96 well plate was then placed in a plastic bag to
447 minimize evaporation and was incubated at 35°C for 20 hours. Penicillin and
448 cefoxitin Etest antimicrobial susceptibility testing on selected MRSA isolates was
449 performed according to manufacturer's guidance. Medium used for Etest was ISA
450 with or without 15 µg/ml clavulanic acid. Etest strips were purchased from bioMérieux
451 UK Ltd.

452

453 **Construction of *S. aureus* gene deletion and complementation mutants**

454 *mecA* deletion mutants in *mecA*-MRSA strains (Supplementary Table 7) were
455 generated by allelic exchange with the temperature-sensitive vector pIMAY, as
456 described previously⁵³. Primers used for *mecA* deletion are listed in Supplementary
457 Table 8. Upstream sequence (AB) and downstream sequence (CD) of the *S. aureus*
458 gene to be deleted were amplified with primers A/B or C/D using KOD Hot Start DNA
459 Polymerase (Merck, UK). PCR products AB and CD were used as templates to
460 obtain deletion construct AD with primers A/D in a splicing overlap extension (SOE)
461 PCR. Product AD was digested with restriction enzymes KpnI and SacI and ligated to
462 pIMAY digested with the same enzymes. The resulting plasmids were designated
463 pIMAYΔ*mecA*. The plasmids were transformed into *E. coli* DC10B (a *dcm* deletion
464 mutant of DH10B), allowing the plasmid to be directly transferred into *S. aureus*
465 strains⁵³. Plasmid DNA extracted from DC10B was then electroporated into recipient
466 strains to create knockout mutants.

467

468 For complement expression of *mecA*, the genes were cloned into expression plasmid
469 pXB01, a derivative of tetracycline-inducible expression vector pRMC2 with the *blaZ*
470 gene deleted⁵⁴. The *mecA* gene variants including the ribosome binding site were
471 amplified from genomic DNA with primers: *mecA*-F-KpnI / *mecA*-R-SacI. PCR
472 products were digested with KpnI and SacI and ligated with the pXB01 vector

cleaved with the same enzymes, generating plasmids *pmecA*_{246E}, *pmecA*_{246G}, and *pmecA*_{122I}. The plasmids were transformed into *E. coli* DC10B, and plasmid DNA then extracted and electroporated into *mecA*-deletion strains for complementation with expression induced with 200 ng/ml Atc.

Antimicrobial susceptibility testing of Danish clinical isolates

Antimicrobial susceptibility testing was performed on a selection of clinical isolates obtained from the Danish surveillance of MRSA and *S. aureus* causing bacteraemia. The selection was based on a total of 270 isolates including 100 isolates (52 MSSA, 48 MRSA) from 2011 on which whole genome sequence data were previously obtained and 170 consecutively received non-CC398 MRSA isolates in 2016. *spa* types were obtained for all isolates. Antimicrobial susceptibility testing was performed as described above except that 0.5 McFarland inoculum was prepared using a densitometer and ISA plates with and without 15 µg/ml clavulanic acid was purchased as custom made plates (SSI Diagnostica, Hilleroed, Denmark).

Expression and purification of PBP2a variants in *E. coli*

PBP2A variants (PBP2a^{E246}, PBP2a^{E246G} and PBP2a^{M122I} from MRSA strains RVC5, BCVA289 and ARARH150, respectively; residues 26-668, with a G26M mutation) were overexpressed using the auto-induction expression method at 25°C⁵⁵. Cells were harvested by centrifugation after 20 hours of expression and lysed using Bugbuster (Novagen, Merck Millipore) containing 10 U/ml of benzonase nuclease (Novagen, Merck Millipore) and Protease Inhibitor Tablets, EDTA-free (Pierce Biotechnology, Thermo Fisher Scientific), following the manufacturer's instructions. After cell disruption, the lysates were cleared, and the soluble proteins were purified using HisPur Ni-NTA Resin columns (Pierce Biotechnology, Thermo Fisher Scientific) under native conditions, according to the manufacturer's instructions. The expression and purification yields were monitored by SDS-PAGE. The most concentrated elution

501 fractions were buffer exchanged to 20 mM sodium phosphate buffer pH 7.4 using
502 PD-10 Desalting Columns (GE Healthcare Life Sciences), following the
503 manufacturer's instructions. Protein concentrations were assessed using the BCA
504 Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific).

505

506 **Bocillin FL PBP2a and penicillin G/clavulanic acid binding assays**

507 The binding affinity of PBP2a for different antibiotics has been determined using a
508 fluorescent penicillin reporter reagent, Bocillin FL⁵⁶⁻⁵⁸. In this study, the affinities of
509 PBP2A variants for penicillin and clavulanic acid were determined using the same
510 approach. Briefly, a reaction mixture containing 25 µg/ml of a purified PBP2a variant
511 in 20 mM sodium phosphate buffer (pH 7.4) and various concentrations (0, 0.5, 1, 5,
512 10, 20, 50, 100, 200, 500, 1000, 2000 µg/ml) of penicillin G or clavulanic acid was
513 incubated at 37°C for 20 min. To test if the presence of clavulanic acid affected the
514 affinity for penicillin G, increasing concentrations of penicillin G together with 15
515 µg/ml of clavulanic acid were assayed. A final concentration of 20 µM Bocillin FL
516 (13.3 µg/ml) was added to the reaction followed by 10 min incubation at 37°C. The
517 reactions were quenched by adding SDS loading buffer and heating at 95°C for 10
518 min. Samples were visualized using 10% Tris-Glycine-SDS PAGE. Protein gels were
519 washed in distilled water for 10 min and scanned using a 473 nm laser of a Fuji
520 Fluorescent Analyzer TLA-5100. Fluorescent intensity was quantified by ImageJ
521 software and IC₅₀ was calculated from three independent assays using GraphPad
522 Prism 5 software.

523

524 **RNA isolation and quantitative real-time PCR (RT-qPCR)**

525 For each selected isolate, 15 ml of early log phase culture with an OD_{595nm} of 0.3 was
526 treated with 10 µg/ml oxacillin for 1 h at 37 °C to induce *mecA* expression and its
527 respective untreated culture was used as a baseline control. After the induction, the
528 OD_{595nm} of the control culture was adjusted to be equal to that of the oxacillin induced

one if necessary and 10 ml of both cultures were spun for 10 min at 4,500 x g at 20 °C. About 0.5 ml supernatant was left behind to re-suspend the pellet and 1 ml of RNAprotect Bacteria Reagent (Qiagen) was added and mixed immediately by vortexing. After incubation for 5 min at room temperature, cultures were spun for 5 min at 10,000 x g at 4 °C to pellet the cells. Pellets were snap-frozen and stored at -80 °C until RNA isolation. Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen) and an additional DNase treatment was performed with the Ambion TURBO DNA-free kit and cDNA was produced using QuantiTect Reverse Transcription Kit (Qiagen). RT-qPCR was performed using the SensiFast SYBR No-ROX Kit (Bioline) on a Rotor-Gene Q machine (Qiagen) using *mecA* and *blaZ* primers. Gene expression fold-changes in induced cultures were calculated relative to control cultures using the $\Delta\Delta C_t$ method⁵⁹ with *gyrB* as the reference.

541

542 **Wax moth larva infection and treatment**

543 The wax moth larvae assay was based on that previously described by Desbois et al
544 ⁶⁰. *Galleria mellonella* larvae were purchased in bulk from Livefood UK. Larvae were
545 stored at 4°C upon arrival and kept at 37°C during the course of the assay. MRSA
546 strains RVC5, 0081, EC139, BCVA289 were selected for evaluation of antimicrobial
547 activities of penicillin and clavulanic acid in combination. Single bacterial colonies
548 were picked to inoculate 5 ml of TSB, and cultures were grown overnight (~16 hours)
549 at 37°C and 200 rpm shaking. Cultures were then diluted 1:100 into 5 ml of fresh
550 TSB and grown for a further 4 hours at 37°C and 200 rpm shaking. Cultures were
551 then centrifuged at 2,500g for 10 minutes, and pellets resuspended in sterile
552 phosphate buffered saline (PBS) to an OD₅₉₅ of 0.2, giving approximately 1.5×10^8
553 CFU/ml. For each strain, six groups of *G. mellonella* (n=10 in each group) were
554 injected with 10 µl aliquots ($\sim 1.5 \times 10^6$ CFU) of resuspended culture behind the rear
555 thoracic segments using a Tridax Stepper Pipette Dispenser (Dymax, UK). Groups of
556 *G. mellonella* were treated by injection with 50 mg/kg vancomycin, 20 mg/kg penicillin

sodium salt, 20 mg/kg clavulanic acid, 20 mg/kg penicillin sodium salt combined with 20 mg/kg clavulanic acid, 20 mg/kg amoxicillin, 20 mg/kg amoxicillin combined with 20 mg/kg clavulanic acid or PBS at 2, 24 and 48 hours after inoculation. The treatments were given blind and the treatment identities not revealed until the experiment was completed. Larvae were considered dead when they did not respond to touch to the head. Survival curves were generated and analysed using GraphPad Prism 6 software. Fig. 5 shows results of a single representative experiment, a replication experiment with broadly similar results is shown in Supplementary Fig. 5.

565

566 **Murine infection model**

567 Fresh overnight colonies from a 5% horse blood agar plate were suspended in saline
568 to an OD₅₄₆ of 0.13, giving approximately 2×10^8 CFU/ml. Mice (NMRI female mice,
569 26 - 30 gram (Taconic, Denmark), 6-8 weeks old were inoculated intramuscularly
570 with 0.05 ml of the suspension in the left thigh (1×10^7 CFU). Approximately 0.5 hrs
571 before inoculation, mice were treated orally with 45 µl Nurofen Junior (20 mg
572 ibuprofen/ml - corresponding to 30 mg/kg) for pain relief. Four mice in each group
573 were treated with a single subcutaneous dose of 0.2 ml with 10, 30 or 100 mg/kg
574 amoxicillin (Amoxil, GlaxoSmithKline Middlesex UK) alone or in combination with 2, 6
575 or 20 mg/kg clavulanic acid (Augmentin, Beecham Group Ltd, Middlesex, UK) or 40
576 mg/kg vancomycin (Fresenius Kabi, Halden, Norway) or saline one-hour post
577 infection. Mice were sacrificed at 1 hour for the start of treatment control group and at
578 5 hours post infection for the treatment groups by cervical dislocation and thighs
579 were collected and kept at -80°C. Each sample thigh was homogenized in 5 ml saline
580 using a Dispomix® Drive, and serially diluted in saline and twenty microliter spots of
581 serial dilutions were plated on blood agar plates. All agar plates were incubated for
582 18 - 24 hrs at 35°C. Statistical comparison was carried out using a 1 way ANNOVA
583 and Dunnett's multiple comparison, was performed for treatment groups comparing
584 against the vehicle group in GraphPad Prism software. All animal procedures were

585 carried out at the Statens Serum Institute (SSI) and approved by the Danish Animal
586 Procedure Inspectorate. Ethical approval was granted for the murine thigh infection
587 model (2016-15-0201-01049). The SSI Animal Welfare Committee (SSI-AWC –
588 equivalent an Institutional Animal Care and Use Committee (IACUC) requires that
589 each experiment is further approved by the supervising laboratory animal
590 veterinarians who are also part of the IACUC. All animals were randomised on arrival
591 at SSI, and sample sizes were based on a combination of statistical analysis and the
592 principles of 3R, that the minimum number of animals were used that were expected
593 to provide statistically significant difference considering the expected intra-group
594 variability of the infection model used.

595

596 **Growth curves**

597 To assess the effect of different *mecA* variants on the growth of MRSA strains in
598 liquid culture, Bioscreen C optical growth analyzer (Lab system, Finland) was used to
599 monitor the growth rates of deletion mutants A75Δ*mecA*, BCVA289Δ*mecA* and
600 EC139Δ*mecA* complemented with the three *mecA* variants. Briefly, overnight
601 cultures were diluted 1/1000 into fresh SSM9PR minimal medium with or without 16
602 µg/ml penicillin supplemented with 200 ng/ml Atc to induce the expression of
603 plasmid-borne *mecA* gene. For each strain, 300 µl of inoculated medium was added
604 into wells of the microplate in triplicate. Fresh medium with Atc was also added to
605 three wells acting as blank controls. Cultures were incubated at 37 °C with
606 continuous shaking for 24 hours and an optical density measurement at OD_{600nm} was
607 taken every 30 mins. Growth curves were analysed using the GraphPad Prism 6
608 software and doubling time calculated using non-linear regression using an
609 exponential growth equation with a least square fit, with Y0 constrained at the
610 minimum optical density measured (an OD_{600nm} of 0.069).

611

612 **Toxicity and biofilm assays**

613 Immortalised human monocyte macrophage THP-1 cell lines were used as described
614 previously⁶¹. Briefly, the cell line was grown in individual 30 mL suspensions of
615 RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 μ M
616 L-glutamine, 200 units/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a
617 humidified incubator with 5% CO₂. Cells were routinely viewed microscopically every
618 48–60 h and harvested by centrifugation at 1,000 rpm for 10 min at room
619 temperature and resuspended to a final density of 1–1.2 x 10⁶ cells/mL in tissue-
620 grade phosphate buffered saline. This procedure typically yielded >95% viability of
621 cells as determined by trypan blue exclusion and easyCyte flow cytometry. To
622 monitor *S. aureus* toxicity, 20 μ L of cells were incubated with 20 μ L of bacterial
623 supernatant and incubated for 12 min at 37°C. For the USA300 strains, supernatants
624 were diluted to 30% of the original volume in TSB as these isolates were
625 considerably more toxic than the single-patient isolates. Cell death was quantified
626 using easyCyte flow cytometry using the Guava viability stain according to
627 manufacturer's instructions. Experiments were done in triplicate, and error bars
628 indicate the average \pm the 95% confidence interval of multiple independent
629 experiments.

630

631 Biofilm formation was quantified using a 1:40 dilution from overnight cultures into 100
632 μ L of fresh TSB supplemented with 0.5% sterile filtered glucose (TSBG) in 96-well
633 polystyrene plate (Costar). Perimeter wells of the 96-well plate were filled with sterile
634 H₂O and plates were placed in a separate plastic container inside a 37°C incubator
635 and grown for 24 h under static conditions. For the transposon mutants, erythromycin
636 (5 μ g/mL) was added to the growth medium. Semi-quantitative measurements of
637 biofilm formation on 96-well polystyrene plates were determined based on the
638 method of Ziebuhr et al²³. Following 24-h growth, plates were washed vigorously five
639 times in PBS, dried and stained with 150 μ L of 1% crystal violet for 30 min at room
640 temperature. Following five washes of PBS, wells were re-suspended in 200 μ L of

641 7% acetic acid, and optical density at 595 nm was recorded using a Fluorimeter plate
642 reader (BMG Labtech). For this experiment the assays were performed in triplicate
643 on each plate and repeated three times.

644

645 **ECOFF determination**

646 In order to help split isolates into phenotypically “wildtype” and “non-wildtype” based
647 on MIC distributions, a series of mixture models were fitted to the data for each drug
648 independently, ranging from 1 to 5 normal distributions. Each model was fitted to the
649 distribution of log-transformed MIC by maximum likelihood. The likelihood function
650 was based on a multinomial distribution over the set of intervals

651 $[0, x_1, \dots, x_n, \infty]$ where x_i stands for each of the tested concentrations for the drug
652 considered. For each drug, the 5 models were compared using Akaike’s Information
653 Criterion to determine the optimal number k of normal distribution components. If the
654 best model was $k=1$, the distribution was labelled as unimodal, and no further
655 analysis was made. For drugs with $k \geq 2$, the following analyses were then carried out:

- 656 1. Predicted distribution of MIC per component: for each component, we multiplied
657 the probability mass at each concentration (= pdf integrated over the immediately
658 lower interval) by the total number of isolates tested.
- 659 2. Direct classification of isolates by component: for each MIC, we split the isolates
660 into the k components according to their relative probabilities at that point.
- 661 3. Optimal cut-off: we then sought to split each distribution into two modes. First, we
662 estimated candidate cut-off points between each successive component of the
663 mixture model. Each cut-off was computed as the concentration that minimised
664 the risk of misclassification of isolates between the model components (achieved
665 by minimising the difference between the cumulative density functions of the two
666 components considered). For example, with $k=3$, there are three components
667 centred at MIC values $Y_1 < Y_2 < Y_3$; we then calculated two candidate cut-offs: one
668 separating component 1 from 2+3, and the other separating 1+2 from 3. For each

cut-off, we then computed the number of isolates that would be misclassified, using the above direct classification as a reference: e.g. for the first cut-off, the number of isolates classified as 2 or 3 with MIC below the cut-off, plus the number of isolates classified as 1 with MIC above the cut-off.

673

674 **Bioinformatics**

Whole genome sequences were assembled using the pipeline described previously⁶². For each isolate the sequence reads were used to create multiple assemblies using VelvetOptimiser v2.2.5⁶³ and Velvet v1.2⁶⁴. The assemblies were improved by scaffolding the best N50 and contigs using SSPACE⁶⁵ and sequence gaps filled using GapFiller⁶⁶. Presence of PBP2a substitutions were identified by extracting the PBP2a sequence from the assembled genome sequences, aligning the PBP2a sequences using Muscle⁶⁷ in Seaview⁶⁸ and then identifying the presence of PBP2a substitutions using a custom python script. The *mecA* promoter mutations were identified using a similar approach using *in silico* PCR to identify the *mecA* promoter region and then aligning the *mecA* promoter sequence and identifying *mecA* mutations using a custom python script. The presence of the *blaZ* genes was confirmed using BLAST against assemblies, this identified that 273/298 of the previously screened WGS isolates had a single copy of *blaZ* (excluding *blaZ*-negative or isolates with two copies or truncated copies of *blaZ*). BlaZ amino acid sequences were then extracted, aligned and amino acids at positions 128 and 216 compared to identify the BlaZ type (Type A: 128:T, 216:S, Type B: 128:K, 216:N, Type C: 128:T, 216:N, Type D: 128:A, 216:S, Type E (LGA251): 128:L, 216:S, Type F (a distinct type identified in this work): 128:A, 216:N) as previously described^{16,69,70}. Phylogenetic analysis of the CC8 isolates was carried out as previously described³³, briefly, sequence reads were mapped using SMALT v0.7.4 (<http://www.sanger.ac.uk/science/tools/smalt-0>) to the *S. aureus* USA300_FPR3757 reference genome (accession: CP000255.1)⁷¹. A core genome alignment was

697 created after excluding mobile genetic element regions, variable sites associated with
698 recombination (detected with Gubbins ⁷²) and sites with more than 5% proportion of
699 gaps (i.e. sites with an ambiguous base). A maximum likelihood (ML) phylogenetic
700 tree was generated with RAxML v8.2.8 ⁷³ based on generalised time reversible
701 (GTR) model with GAMMA method of correction for among site rate variation and the
702 phylogenetic tree annotated using Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).
703 Phylogenetic analysis of PBP2a sequences was constructed by using PhyML v3.0 in
704 Seaview with a Whelan and Goldman (WAG) substitution model and 100 bootstrap
705 replicates ⁷⁴.

706

707

708 **Population analysis**

709 Population analysis was carried out as described by Kim et al ⁷⁵ with minor
710 modifications. Strains were grown overnight in tryptic-soya broth (TSB), and serial
711 10-fold dilutions were plated in triplicate onto Iso-Sensitest Agar (Oxoid) plates
712 containing varying concentrations of penicillin with 15 µg/ml clavulanic acid. Plates
713 were incubated for 24 hours at 35°C. A mean of the three platings were plotted in
714 Figure 5a. Biological replicates for strains BCVA289 and 0081 were generated and
715 plotted in comparison to the original results in Supplementary Figure 4, showing
716 broadly similar results.

717

718 **Structural modelling of PBP2a**

719 Co-ordinates from representative structures of both the Gly246 (accession code
720 3ZFZ, ²¹) and Glu246 (accession code 1VQQ, ⁷⁶) forms of PBP2a were used to
721 compare possible effects induced by sidechain alteration. Figures were prepared
722 using Chimera ⁷⁷ and Pymol (The PyMOL Molecular Graphics System, Version
723 1.2r3pre, Schrödinger, LLC).

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998 **Correspondence and requests for materials should be addressed to EMH.**

999

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1015

1016 **Author contributions**

1017 Design of the study: EMH, XB, SJP, MAH. *mecA* deletion and complementation,
1018 expression analysis, bocillin assays: XB. Antimicrobial susceptibility testing: XB, BB,
1019 NG, KB. Biofilm and toxicity assays: HC and RM. Antimicrobial susceptibility testing
1020 of Danish isolates: JL, ARL. Determination of the ECOFF: OR. Structural analysis of
1021 PBP2a: AL. Infection and treatment experiments: EMH, XB, CVL. Bocillin binding
1022 assays: IRG and RGS. Bioinformatics analysis of whole-genome sequence data:
1023 EMH, FC, SR, DJ. Collection of USA300 isolates: ACU, FDL. Wrote bioinformatics
1024 scripts: NG. Analysis and interpretation of the data: CUK, GP, MTGH, JP.
1025 Coordinated the study and wrote the manuscript: EMH. Responsible for supervision
1026 and management of the study: SJP and MAH. All authors read, contributed to and
1027 approved the final manuscript.

1028 **Competing interests**

1029 CUK is a consultant for the World Health Organization (WHO) Regional Office for
1030 Europe, QuantuMDx Group Ltd, and the Foundation for Innovative New Diagnostics,
1031 which involves work for Cepheid Inc., Hain Lifescience and WHO. CUK is an advisor
1032 to GenoScreen. The European Society of Mycobacteriology awarded CUK the
1033 Gertrud Meissner Award, which is sponsored by Hain Lifescience. The Bill & Melinda
1034 Gates Foundation, Janssen Pharmaceutica, and PerkinElmer covered CUK's travel
1035 and accommodation to present at meetings. The Global Alliance for TB Drug
1036 Development Inc. and Otsuka Novel Products GmbH have supplied CUK with
1037 antibiotics for *in vitro* research. CUK has collaborated with Illumina Inc. on a number
1038 of scientific projects. SJP and JP are consultants to Next Gen Diagnostics Llc. SJP is
1039 a consultant to Specific Technologies. All other authors declare no competing
1040 financial interest

1041

1042 **Data availability**

1043 All data generated or analysed during this study are included in this published article
1044 (and its supplementary information files).

1045

1046 **Figure Legends**

1047 **Figure 1: Penicillin susceptibility in the presence of clavulanic acid.** (a) Figure
1048 shows a representative image of two MRSA isolates grown on Iso-sensitest agar or
1049 Iso-sensitest agar with 15µg/ml of clavulanic acid with a penicillin E-test strip applied,
1050 the upper susceptible isolate shows increased susceptibility to penicillin in the
1051 presence of clavulanic acid, while the lower resistant isolate shows only a minor
1052 effect. (b) E-test determination of minimum inhibitory concentration of penicillin alone
1053 (black) and penicillin plus 15 µg/ml clavulanic acid (grey) of a selection of penicillin-
1054 clavulanic acid susceptible (n=14) and resistant (n=10) MRSA isolates. The red line
1055 indicates the current EUCAST clinical break point of ≤ 0.125 µg/ml. The effect of
1056 clavulanic acid on susceptibility as measured by disk diffusion to different β -lactam
1057 antibiotics in a panel of (c) penicillin-clavulanic acid resistant MRSA isolates (n=46),
1058 and (d) penicillin-clavulanic acid susceptible MRSA isolates (n=32). Red bars
1059 indicate isolates grown on Iso-Sensitest Agar (ISA) alone and blue bars indicate
1060 isolates grown on ISA supplemented with 15µg/ml clavulanic acid. Y-axis indicates
1061 the zone of inhibition in millimetres. Error bars indicate the standard deviation of the
1062 mean. Note: OX(MHA) = Oxacillin performed on Müller-Hinton agar (recommended
1063 media by EUCAST and CLSI).

1064

1065 **Figure 2: PBP2a substitutions mediating penicillin susceptibility.** (a) Minimum
1066 inhibitory concentrations (MIC) of penicillin (black) or penicillin in the presence of 15
1067 µg/ml clavulanic acid (grey) for wildtype strains EC139 (ST398) and BCVA289

1068 (USA300), and *mecA* mutants and complemented mutant strains with either an
 1069 empty vector (p) or one of the three different alleles of *mecA* (PBP2a^{246E} (p246E),
 1070 PBP2a^{246G} (p246G), and PBP2a^{112I} (p112I)). Results presented are the mean of three
 1071 independent experiments. **(b)** Bocillin competition assay to determine the IC₅₀ of
 1072 penicillin for the three PBP2a variants (PBP2a^{246E} – ‘WT’, PBP2a^{246G} and
 1073 PBP2a^{M122I}). **(c)** Bocillin competition assay to determine the IC₅₀ of penicillin in the
 1074 presence of 15µg/ml clavulanic acid for the three PBP2a variants (PBP2a^{246E} – ‘WT’,
 1075 PBP2a^{246G} and PBP2a^{M122I}). The fluorescence intensity of the bands was plotted as
 1076 the percentage of unbound proteins as a function of penicillin G concentration, and
 1077 the IC₅₀ value was calculated from the plot. Data points represent the average of
 1078 three replicates and the curve is the predicted nonlinear regression result. **(d)** Mixture
 1079 models of MIC distributions of 298 MRSA isolates for penicillin in the presence of 15
 1080 µg/ml clavulanic acid, the best model is a mixture of 4 normal distributions, with a
 1081 proposed cut-off at 2.449, and **(e)** for penicillin alone, the best model is a mixture of 4
 1082 normal distributions, with a proposed cut-off at 0.218. The graphs show the proposed
 1083 categorisation of the MIC distribution into “wildtype” isolates (low MIC, green hues)
 1084 and “non-wildtype” isolates (high MIC, amber hues), and the proposed empirical cut-
 1085 off as the vertical dashed line. Amber isolates that fall below the cut-off and green
 1086 isolates that fall above the cut-off show the expected classification errors by applying
 1087 the cut-off.

1088

1089 **Figure 3: Genetic basis of MRSA penicillin/clavulanic acid susceptibility. (a)**
 1090 Summary of PBP2a substitutions and *mecA* promoter mutations. Figure shows a
 1091 representation of the domain structure of the PBP2a protein and *mecA* promoter with
 1092 the location of the two PBP2a substitutions and *mecA* promoter associated with
 1093 penicillin susceptibility indicated and mean penicillin MIC in the presence of 15 µg/ml
 1094 of clavulanic acid for isolates with that genotype. **(b)** Relative *mecA* expression
 1095 measured by RT-qPCR. Figure shows the relative *mecA* expression after oxacillin

induction, normalised to *gyrB* for isolates with *mecA*[-7]:T (n=3), *mecA*[-7]:G (n=7) and *mecA*[-33]:T (n=6). Error bars indicate the standard deviation of the mean. Data were analysed with a two-tailed, unpaired t-test. * $P = 0.0048$, ** $P = 0.0016$. **(c)** MIC distributions for penicillin (upper graph) and penicillin with 15 µg/ml clavulanic acid (lower graph) with the number of isolates with each genotype combination of PBP2a substitutions and *mecA* promoter mutations - indicated by colouring of the histogram bars. **(d)** Joint distribution of MIC for 298 MRSA isolates, x axis shows penicillin MIC and y axis shows penicillin MIC in the presence of 15 µg/ml clavulanic acid. Colour and shape of the plot points indicate the genotype of the isolate.

Figure 4: Prevalence and population genomics of penicillin-clavulanic acid.

(a) Graph shows the percentage abundance in the overall population and by clonal complex (CC) of penicillin-clavulanic acid susceptible and resistant genotypes in 2282 clinical MRSA isolates from Cambridge, UK **(b)** Presence of *SCCmec* elements in clonal complex (CC)8 and USA300 isolates. Figure shows a maximum likelihood phylogenetic tree constructed from core genome SNPs of 580 CC8 isolates. USA300 isolates are indicated by blue colouring of the branches. Tips of the tree indicate the *SCCmec* type (red = IVa, blue = IVb = orange = IVc, pink = IVd, purple = IVg, NT (light blue) = non-typeable, - = negative for *SCCmec*). Isolates that were phenotypically tested for penicillin-clavulanic acid susceptibility are indicated with an asterisk. Additionally, isolates: BCVA289, which was used for *in vivo* testing (wax moth) and 43484, which was used for *in vivo* testing (murine thigh) are included for comparison.

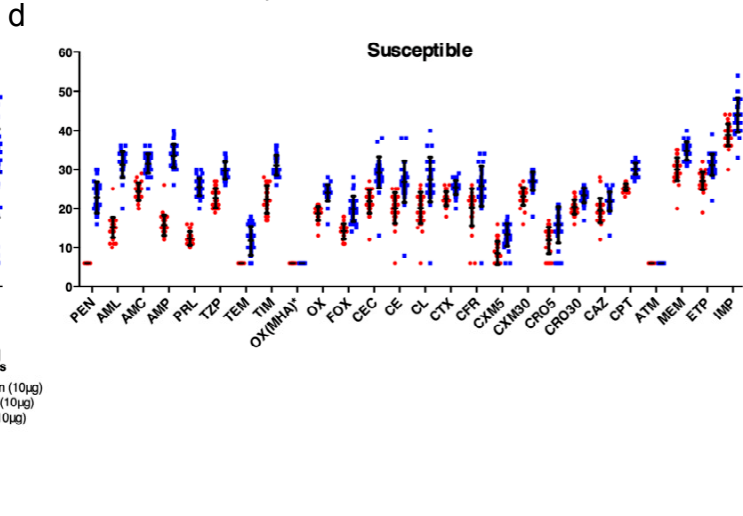
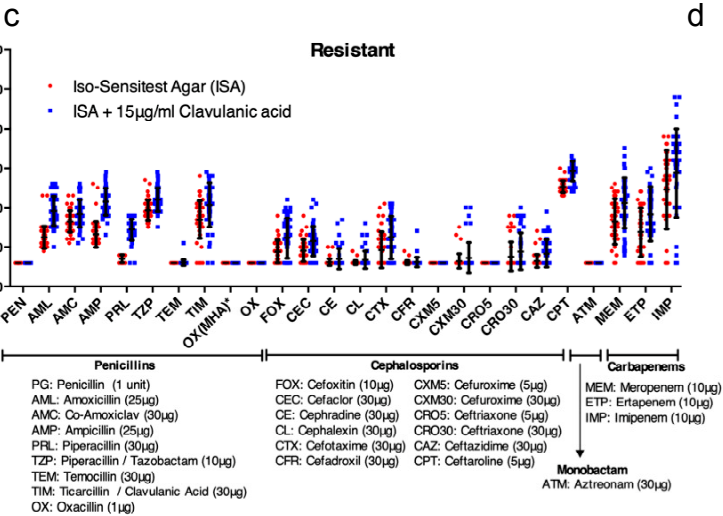
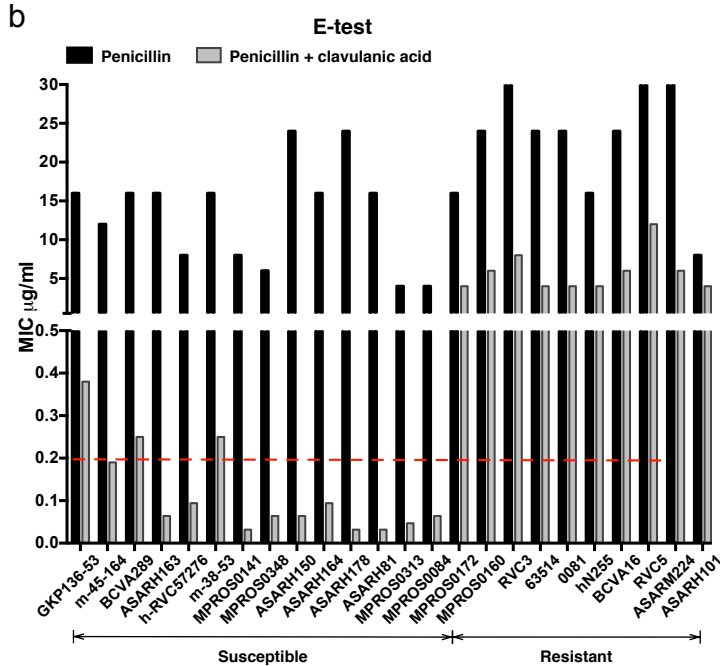
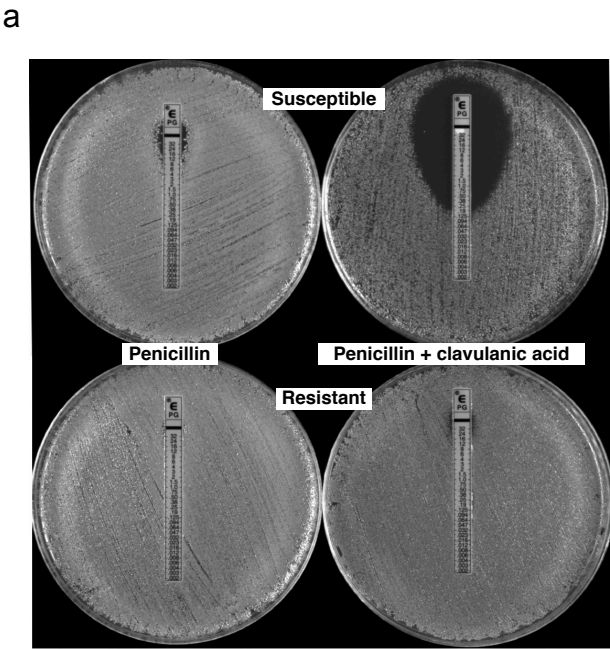
Figure 5: Penicillins and clavulanic acid are efficacious for the treatment of susceptible MRSA. (a) Population analysis of resistance to penicillin and clavulanic acid. Figure shows the log₁₀ CFU/ml of the different strains at various concentrations of penicillin G (µg/ml) in the presence of 15 µg/ml clavulanic acid on Isosensitest

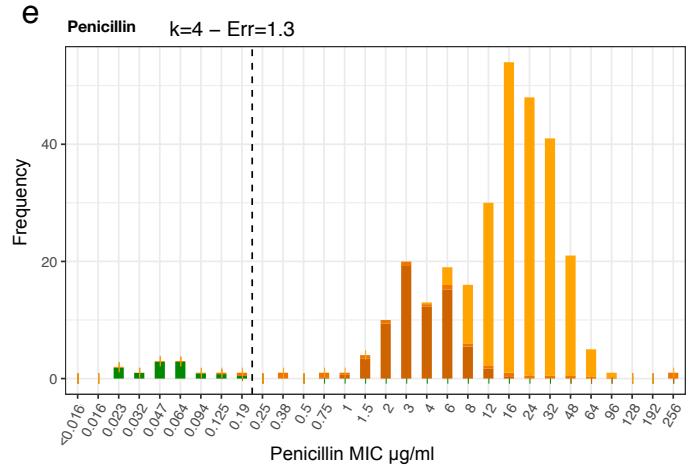
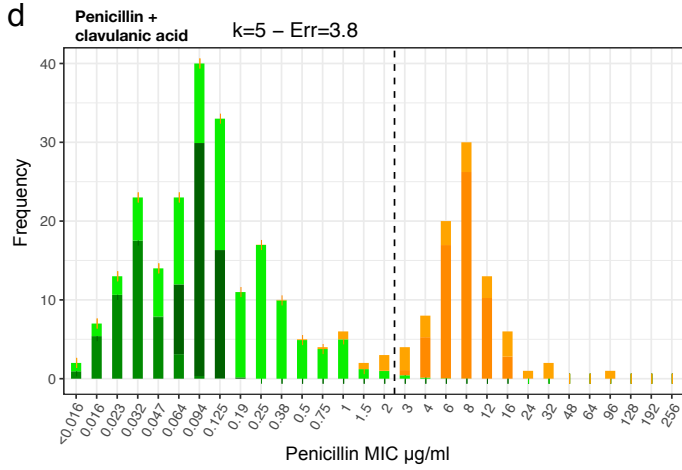
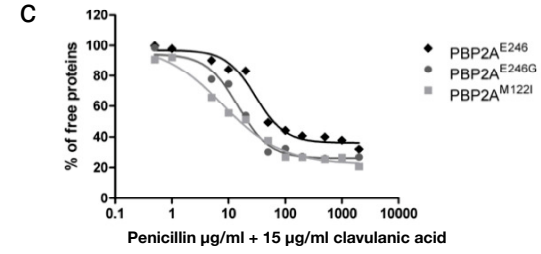
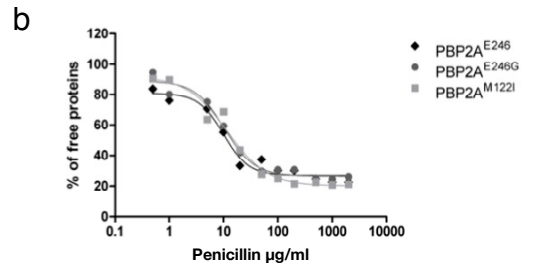
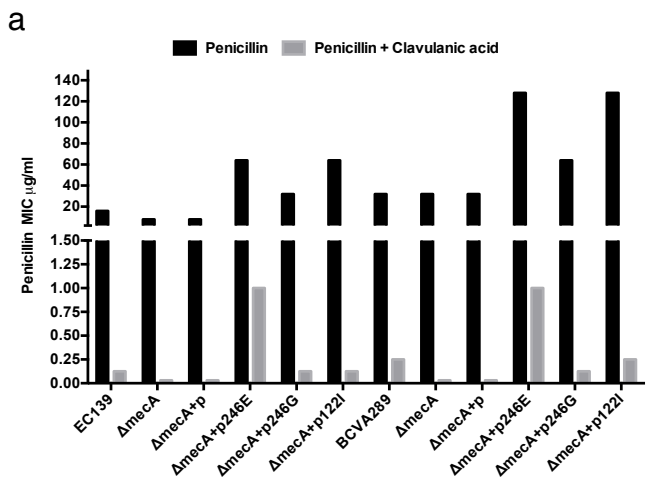
1124 plates. Plotted points are the mean of three technical replicates (biological replicates
 1125 for BCVA289 and 0081 shown in Supplementary Fig. 4). Survival curves for wax
 1126 moth larvae (*Galleria mellonella*) infected with $\sim 1.5 \times 10^6$ CFU of: **(b)** 0081 (ST22)
 1127 (penicillin-clavulanic acid MIC = 4 μ g/ml - resistant), **(c)** RVC5 (ST22) (MIC = 12
 1128 μ g/ml - resistant), **(d)** BCVA289 (ST8 - USA300) (MIC = 0.25 μ g/ml - susceptible),
 1129 and **(e)** (EC139 (ST398) (MIC = 0.25 μ g/ml - susceptible). Ten larvae in each group
 1130 were experimentally infected and then treated at 2, 24, and 48 hours with
 1131 vancomycin (50 mg/kg), penicillin (20 mg/kg) clavulanic acid (20 mg/kg), penicillin-
 1132 clavulanic acid (1:1 ratio - 20 mg/kg), amoxicillin (20 mg/kg), amoxicillin-clavulanic
 1133 acid (1:1 ratio - 20 mg/kg) and PBS alone. Presented data are from a single
 1134 representative experiment, a replication experiment is presented in Supplementary
 1135 Fig. 5. **(f)** Effect of amoxicillin alone and in combination with clavulanic acid against
 1136 MRSA in a murine thigh infection model. Four mice in each treatment group were
 1137 inoculated with $7 \log^{10}$ CFU of MRSA strain 43484 (USA300) and treated 1-hour post
 1138 inoculation with a single subcutaneous injection of either vancomycin (40mg/kg),
 1139 Amoxicillin (Amox) (10, 30, 100 mg/kg) alone or in combination (5:1 ratio) with
 1140 clavulanic acid (Clav) (2, 6, 20 mg/kg). The bar indicates the mean. **** indicates a
 1141 significant difference ($p < 0.0001$, Dunnett's multiple comparisons test) between
 1142 vehicle control. NS indicates there was no significant difference ($p = 0.0982$, Dunnett's
 1143 multiple comparisons test) between the combined amoxicillin 100 mg/kg: clavulanic
 1144 acid 20mg/kg and vancomycin 40 mg/kg.

1145

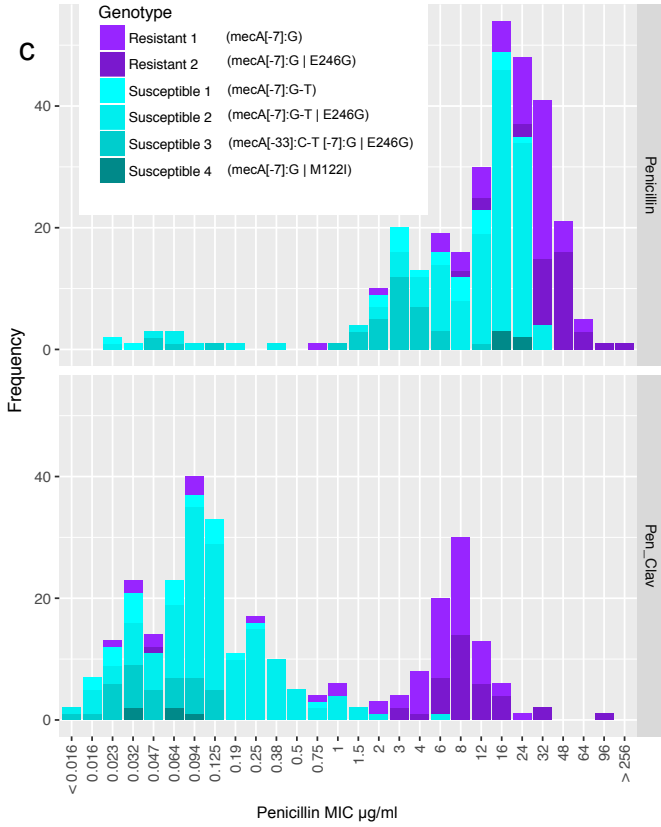
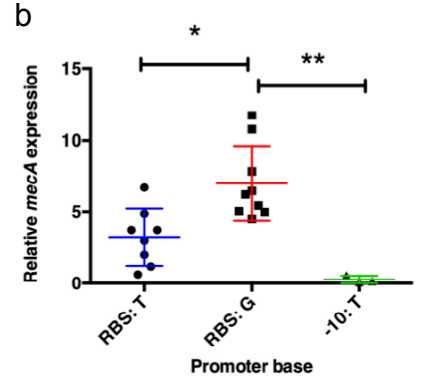
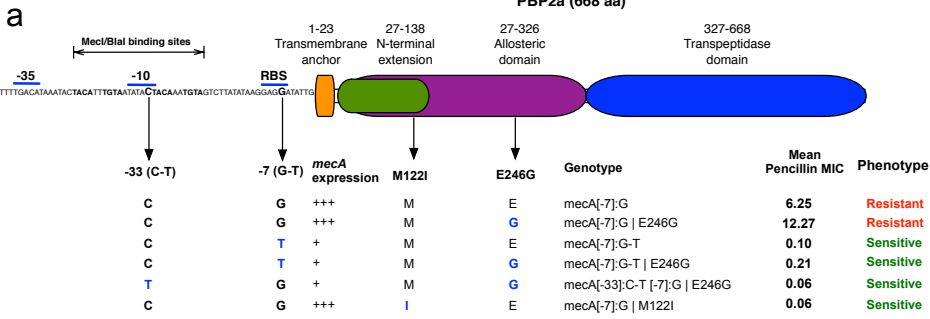
1146 **Figure 6: PBP2a^{246G} substitution provides an increased growth rate in the**
 1147 **presence of penicillin.** Figure shows growth curves for strain BCV289 Δ *mecA* grown
 1148 in **(a)** SSM9PR minimal medium and **(b)** SSM9PR with 16 μ g/ml penicillin, strain
 1149 EC139 Δ *mecA* grown in **(c)** SSM9PR minimal medium and **(d)** SSM9PR with 16
 1150 μ g/ml penicillin, and strain A75 Δ *mecA* grown in **(e)** SSM9PR minimal medium and **(f)**
 1151 SSM9PR with 8 μ g/ml penicillin. Lines are coloured depending on the vector the two

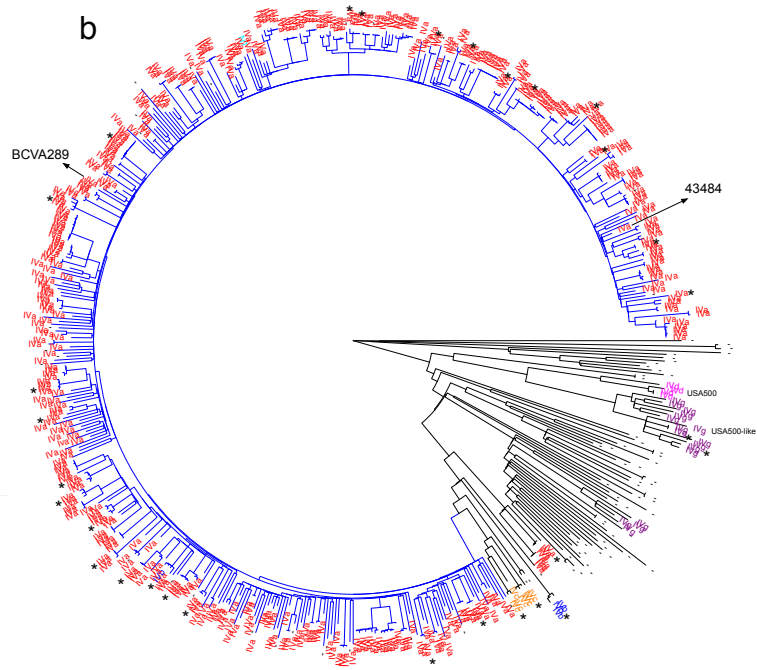
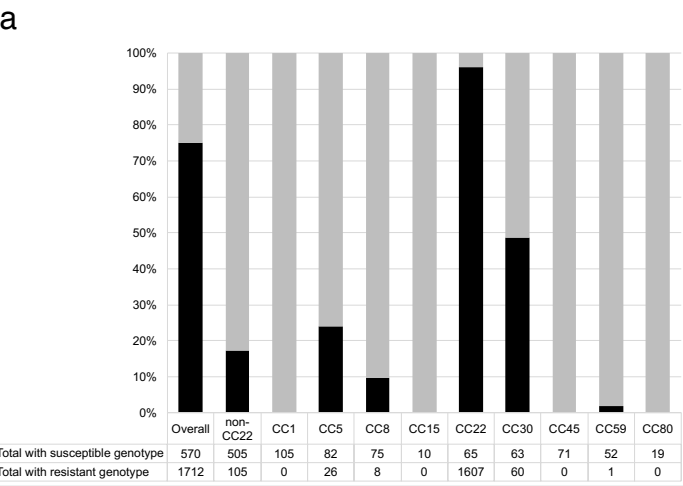
1152 strains were complemented with: orange = vector only control, green = vector
1153 expressing *mecA*^{246G}, blue = vector expressing *mecA*^{246E}, black = vector expressing
1154 *mecA*^{122I}. The mean of a minimum of 6 independent replicates are plotted and error
1155 bars indicate standard deviation.
1156

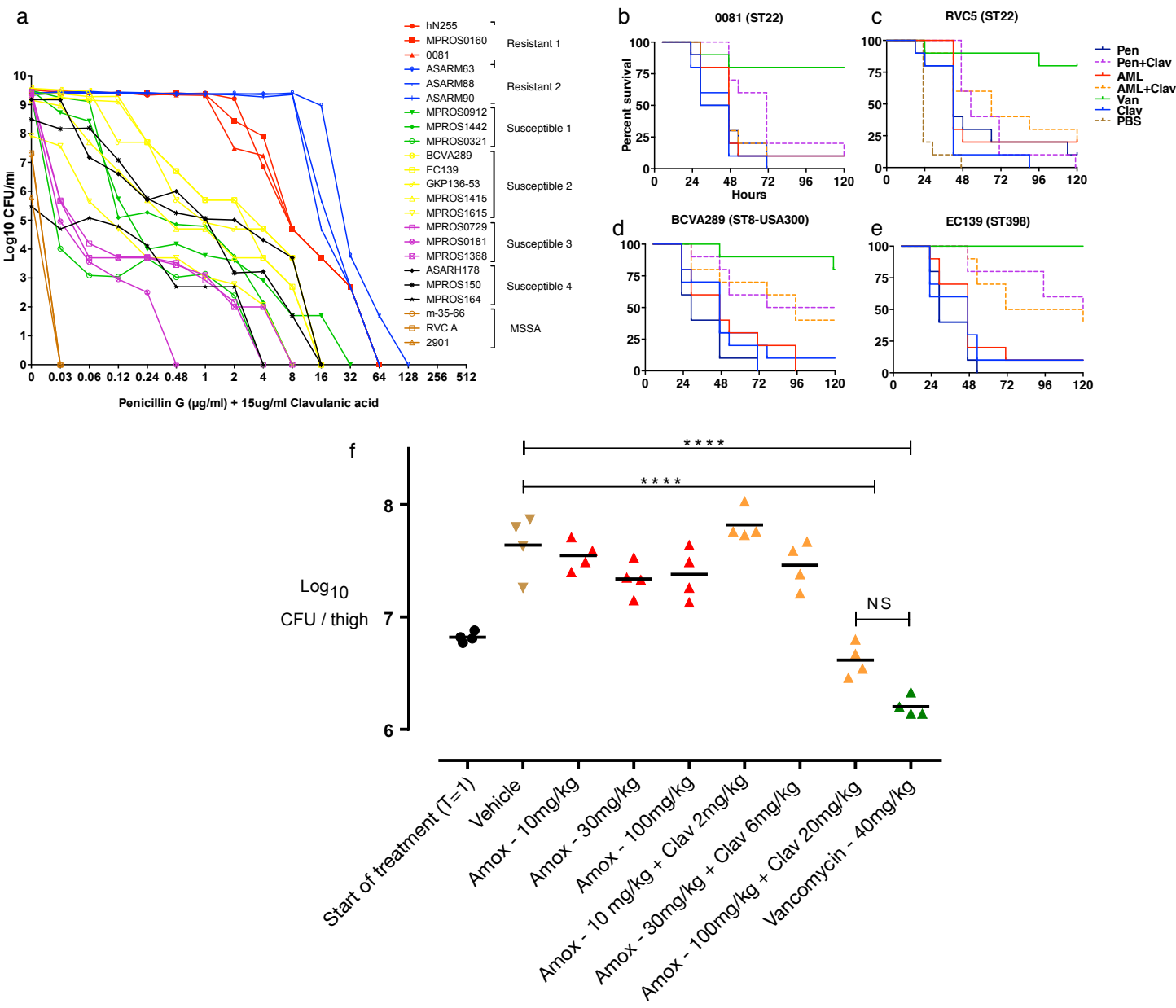




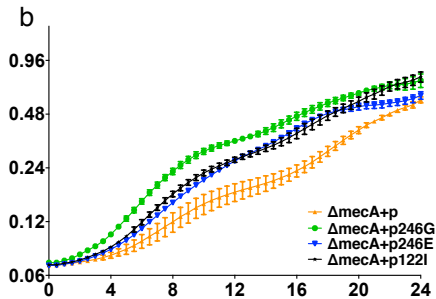
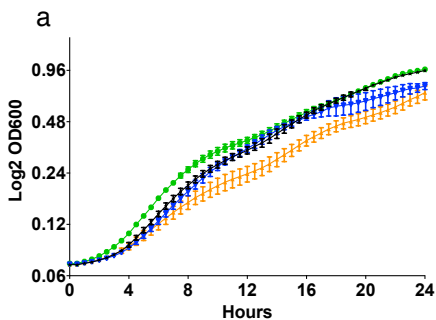
PBP2a (668 aa)



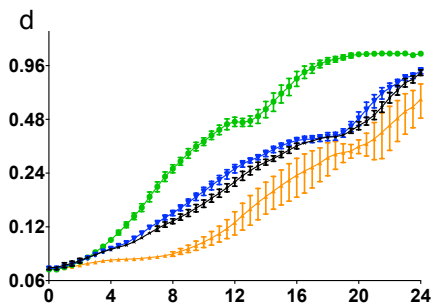
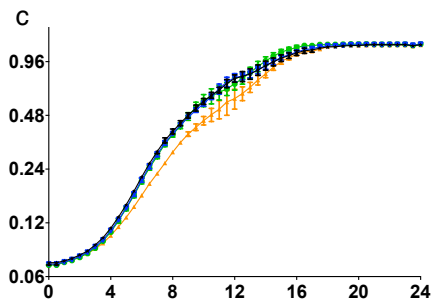




BCVA289 (ST8-USA300)



EC139 (ST398)



A75 (ST22)

